Gladiolic Acid, an Antibiotic Substance Produced by Penicillium gladioli McCull. & Thom

By P. W. BRIAN, P. J. CURTIS AND H. G. HEMMING

Imperial Chemical Industries Ltd., Butterwick Research Laboratories, Welwyn, Herts

SUMMARY: Gladiolic acid is produced when Penicillium gladioli is grown on a wide range of culture media. The main factor influencing its production and accumulation is the pH drift of the medium, high yields being associated with a characteristic pH drift, consisting of an initial fall to about pH 4.0 followed by a steady but not too rapid rise. Continued low pH is unfavourable; too rapid a rise of pH is also unfavourable since gladiolic acid tends to disappear from the medium when pH 6.0 is reached, the disappearance being very rapid at pH 7.0 or above. The effects of variation of the initial pH of the medium, of glucose concentration, of variation of nitrogen source and of additions of certain organic acids are all explicable in terms of their effect on pH drift.

The antibiotic is best extracted from culture filtrates by treatment with activated charcoal after adjustment to pH 4.0, elution of the charcoal with ether, and re-crystallization from water after evaporation of the ether. Yields of the order of 300 mg./l. are obtained.

Gladiolic acid is highly fungistatic if tested at low pH; the toxic effect is due to the undissociated molecules only and at pH 7.0, when dissociation is virtually complete, gladiolic acid is almost inactive. At pH 8.5, the least concentration inhibiting germination of fungus spores varies from 0.9 µg./ml. for Fusarium graminearum to 250 µg./ml. for Trichoderma viride. It is not highly antibacterial in broth, many organisms growing freely in the presence of 500 µg./ml. This low activity is thought to be due in part to the dissociation of gladiolic acid at pH 7.0 and in part to inactivation by certain broth constituents. This view is supported by the observation that bacterial cells suspended in gladiolic acid solutions (100 µg./ml.) in buffer at pH 4.0 are rapidly killed. This bactericidal effect occurs with both Gram-positive and Gram-negative organisms.

Gladiolic acid in solution is relatively stable in the range pH 3.0-8.0. In the presence of ammonium salts or certain amino-acids, notably p-aminobenzoic acid, it is rapidly inactivated, coloured complexes being formed. The reaction with ammonium salts is dependent on pH, not proceeding at pH 3.5 but proceeding rapidly at pH 7.0. The rapid disappearance of gladiolic acid in culture when the pH rises above 6.0 is possibly associated with this type of reaction.

Penicillium gladioli McCull. & Thom is found in many parts of the world as a weak parasite on Gladiolus corms in storage (Moore, 1939). It is uncertain whether infection of the corm takes place in the soil or at a later stage, but it seems certain that on some occasions infection takes place before the corms are lifted; this suggests that the fungus may be free-living in the soil, though no record exists of its direct isolation from soil.

In a preliminary communication (Brian, Curtis, Grove, Hemming & McGowan, 1946) strains of P. gladioli were shown to produce a strongly antifungal and weakly antibacterial substance, gladiolic acid, which appeared to be a methoxy methyl 2-carboxyphenyl glyoxal (C11 H16 O6). The present communication is confined to a more detailed study of the conditions of production of gladiolic acid and of its biological properties; its chemistry will be dealt with in a separate publication.
Assays of fungistatic and bacteriostatic activity. For routine assays of fungistatic activity a spore germination test has been used, with conidia of Botrytis allii Munn. The results are expressed in arbitrary B.A. units/ml; a B.A. unit is that quantity of antibiotic which, dissolved in a stated quantity of Weindling medium, reduces germination of B. allii spores to 2% or less. Details of this technique have been previously described (Brian & Hemming, 1945). Unless otherwise stated, all solutions were adjusted to pH 3.5 before assay of fungistatic activity. Assays of bacteriostatic activity were made by serial dilutions in broth. Other special methods, where used, are described later.

Methods of culture. In experiments which did not involve extraction of gladiolic acid, cultures were grown on 250 ml lots of medium in 'Glaxo' culture vessels (Clayton, Hems, Robinson, Andrews & Hunwicke, 1944). Samples of the underlying medium were withdrawn periodically for assay, under sterile conditions. In general, six cultures were set up on each medium in an experiment, the samples for each vessel in each set of six being bulked for assay. For bulk production, earthenware culture vessels, each holding 0.5-1 l., were used.

A crude grade of glucose was used in all media unless otherwise stated. All cultures were grown at 25°.

Production of spores for inoculum. Sporulation of P. gladioli is closely dependent on temperature. McCulloch & Thom (1928) record that at 20° few conidiophores are produced, though sclerotia are formed abundantly, whereas at 15° conidiophores and conidia are produced freely, with few sclerotia. Gladiolic acid is produced by both of the two strains of P. gladioli examined: no. 59 (N.C.T.C. 3994), isolated by F. T. Brooks in 1931, can be characterized as a conidial strain, producing conidia abundantly at room temperature (c. 15-20°), with few sclerotia; no. 206, isolated from a Gladiolus corm in 1944, can be characterized as a sclerotial strain, producing sclerotia abundantly, with very few conidiophores, even at room temperature. Since easy production of spores for inoculum was a matter of importance, strain no. 59 was used in all the experiments now described. It was found best to grow the mould on Czapek-Dox agar in flat medicine bottles; these are incubated for 3-4 days at 25° and the bottles are then removed from the incubator and kept at room temperature. Under these conditions conidia are produced abundantly, though few are produced if the cultures are incubated at 25° throughout. Conidia were removed from the cultures by adding a little sterile water to the bottle and gently rubbing the agar with a sterile glass rod.

Relation between medium and development of fungistatic activity

Preliminary experiments indicated that Raulin-Thom medium was more effective than Czapek-Dox, somewhat higher titres being attained and maintained for several days, whereas on Czapek-Dox a sharp decline followed the development of peak activity. Accordingly, production of gladiolic acid in a medium of the Raulin-Thom type was studied, and in later stages, in
Gladiolic acid

a simplified medium. The gladiolic acid was not extracted, but was assayed by the *B. allii* spore-germination test. It was assumed that all activity was due to gladiolic acid. Our experience in extraction experiments has justified this assumption.

The variables studied included initial pH of the medium, concentration of carbon source (glucose), type of nitrogen source, and the effect of additions of malic acid to ammonia-nitrogen media. All the experimental data presented below indicate that these factors mainly influence the pH drift of the medium during growth of the mould.

Table 1. **Effect of initial pH of Raulin-Thom medium (7·5% (w/v) glucose)**

<table>
<thead>
<tr>
<th>Initial pH</th>
<th>4</th>
<th>8</th>
<th>11</th>
<th>15</th>
<th>21</th>
<th>Mean final dry wt. of mycelium (g./culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3·2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2·7</td>
</tr>
<tr>
<td>4·2</td>
<td></td>
<td>12</td>
<td>32</td>
<td>48</td>
<td>48</td>
<td>3·0</td>
</tr>
<tr>
<td>5·0</td>
<td>8</td>
<td>64</td>
<td>24</td>
<td>24</td>
<td>4</td>
<td>3·3</td>
</tr>
<tr>
<td>6·1</td>
<td>6</td>
<td>96</td>
<td>32</td>
<td>32</td>
<td>6</td>
<td>3·1</td>
</tr>
<tr>
<td>6·8</td>
<td>6</td>
<td>96</td>
<td>24</td>
<td>24</td>
<td>4</td>
<td>3·1</td>
</tr>
</tbody>
</table>

*Initial pH of Raulin-Thom medium.* Raulin-Thom medium was prepared, according to the formula given by Brian, Curtis & Hemming (1946), with initial pH ranging from 3·2 to 6·8. The glucose and salts were made up in separate solutions, the pH of the salt solution being adjusted with HCl or KOH before autoclaving; and the two sterile solutions mixed under sterile conditions. This procedure avoids the considerable breakdown of glucose that occurs if autoclaved in the complete medium at high pH. Results of periodical assays and final dry weights of the mycelium from *P. gladioli* cultures on these media are recorded in Table 1.

It will be seen that the low initial pH does not favour the production of fungistatic substances, none developing in the medium initially adjusted to pH 3·2 and some developing only slowly in the medium initially adjusted to pH 4·2. Growth and development of the fungus were also affected; on the pH 3·2 medium the mycelial felt produced was never completely confluent and few, if any, conidia were formed. At pH 4·2 growth was confluent, but sporulation was reduced. At higher pH values thick, vigorously sporing felts were rapidly produced. The final dry weight of mycelium (i.e. after 21 days' growth) was not very markedly affected by initial pH, though the same general trend was observed; the failure to produce gladiolic acid in media of low pH cannot be attributed to reduction in the growth rate of the mould. Study of the pH drift (Fig. 1) reveals a significant difference between the media rapidly producing high concentrations of gladiolic acid and the two poorer ones. The pH 3·2 medium shows an initial fall to pH 2·0 followed by a slow rise to pH 2·6. The pH 4·2 medium shows an initial fall to pH 3·0 followed by a rise to pH 5·1 and the fungistatic activity develops during the phase of rising pH. The
remaining three media (pH 5·0, 6·1, 6·8) show similar pH drifts; there is an initial fall to near pH 3·0, followed by a rapid rise to pH 6·0-6·5 after which a fairly steady level is maintained. Fungistatic activity develops rapidly during the phase of rising pH, but then falls. The significance of these relations becomes clearer after consideration of further experiments.

![Figure 1: Effect of initial pH of Raulin-Thorn medium on pH drift in cultures of Penicillium gladioli.](image)

**Table 2. Effect of glucose content of Raulin-Thorn medium (pH 5·5) on fungistatic activity (B.A. units/ml.) of Penicillium gladioli cultures**

<table>
<thead>
<tr>
<th>Dextrose (w/v)</th>
<th>Mean final dry wt. of mycelium (g./culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1·0</td>
<td>2·1</td>
</tr>
<tr>
<td>2·5</td>
<td>3·4</td>
</tr>
<tr>
<td>5·0</td>
<td>3·1</td>
</tr>
<tr>
<td>7·5</td>
<td>4·8</td>
</tr>
<tr>
<td>10·0</td>
<td>4·7</td>
</tr>
<tr>
<td>15·0</td>
<td>4·6</td>
</tr>
</tbody>
</table>

**Glucose concentration in Raulin-Thom medium.** *P. gladioli* was grown on Raulin-Thom medium made up, at pH 5·5, with glucose concentrations ranging from 1·0 to 15·0% (w/v). Results of assays, pH drift and mycelial weights are recorded in Table 2 and Figs. 2 and 3. This experiment is particularly illuminating. The following points should be noted.

(a) The typical pH drift is an initial fall followed by a rise, the rate of rise in pH being inversely proportional to the glucose concentration. Thus, for example, the medium with 15·0% falls to pH 3·5 in 4 days and thereafter rises slowly to pH 5·8 after 20 days; the medium with 2·5% glucose falls to pH 4·4 in 4 days and thereafter rises rapidly, reaching pH 7·0 in a further 2 days,
Fig. 2. Relation between glucose concentration and pH drift in cultures of *Penicillium gladioli* on Raulin-Thorn medium. (×, 1·0 % glucose; Δ, 2·5 %; ○, 5·0 %; □, 7·5 %; +, 10·0 %; ▲, 15·0 %.)

Fig. 3. Relation between glucose concentration and development of fungistatic activity in cultures of *Penicillium gladioli* on Raulin-Thorn medium. (Δ, 2·5 % glucose; ○, 5·0 %; □, 7·5 %; +, 10·0 %, ▲, 15·0 %.)
after which the change in pH is more gradual. The medium with only 1.0 % glucose is anomalous to the extent that no initial fall in pH was recorded, though this might have been detected if the medium had been tested earlier.

(b) Initially, the rate of development of fungistatic activity (neglecting for the moment the medium with 1.0 % glucose) is inversely related to the glucose concentration.

(c) The peak of fungistatic activity is directly related to the glucose concentration, a slow initial rise in assay being correlated with a higher peak assay.

(d) Fungistatic activity, after reaching a maximum, falls off in all media save that with 15.0 % glucose. The fall in activity in each case coincides with the development in the medium of a pH above 6.0. The absence of a fall in activity in the medium with 15.0 % glucose is consistent with the fact that at the end of the experiment the critical pH 6.0 had not been reached.

(e) Although with increasing glucose concentration the weight of mycelium formed increases, the difference in mass of mycelium cannot be held to account for the differences in gladiolic acid assay.

These experiments on the effect of glucose concentration and initial pH strongly suggest that an upward trend of pH in the medium favours gladiolic acid production or accumulation, but that once pH 6.0 is reached the gladiolic acid is progressively destroyed. This view was confirmed by an experiment using different carbon sources. The fungus was unable to utilize lactose; little growth took place and no gladiolic acid was produced. Starch, dextrin, sucrose, glucose and glycerol were all utilized; the pH drift with glycerol and sucrose was much more gradual than with glucose and higher assays were maintained much longer with these two materials. In each case activity began to fall when pH 6.0 was reached.

*Nitrogen source and organic acid supplement.* In an experiment with a number of different nitrogen sources a simplified medium (Brian, Curtis & Hemming, 1947) was used. The nitrogen sources were potassium nitrate, ammonium nitrate, ammonium sulphate and ammonium tartrate. Experience with other fungi suggested that at times the whole course of metabolism may differ if ammonia nitrogen is substituted for nitrate nitrogen. There is also evidence (Brian et al. 1947) that utilization of ammonia nitrogen may be facilitated by the presence of certain organic acids and hence ammonium tartrate was compared with ammonium sulphate; supplements of malic acid (0.5 % w/v) to ammonium sulphate media were tested for similar reasons.

The various nitrogen sources were added to give nitrogen equivalent to 0.28 % potassium nitrate, at two glucose levels (2.5 and 10.0 %), the media being adjusted to pH 5.5. *P. gladioli* was grown on these media in the usual way. It is quite clear from these data (Table 3 and Fig. 4) that the form of nitrogen supplied is of little importance except in so far as it affects the drift of pH in the medium. Ammonium sulphate alone failed to give high assays with either low or high glucose concentrations. This is probably explained by the uniform low pH of ammonium sulphate media, previously shown to be unfavourable to gladiolic acid production; in the series with high sugar concentration growth in ammonium sulphate media was relatively reduced, but
not to a sufficient extent to explain the low assays. Addition of malate to ammonium sulphate media led to an upward pH drift and correspondingly higher assays. With all the media the assay rose with a rising pH drift in the medium, falling when a pH above 6.0 was reached. The results are of interest, too, in showing that a low glucose concentration (2.5%) is not intrinsically unfavourable; in a medium with ammonium tartrate as nitrogen source, in

Table 3. Effect of nitrogen source, at two glucose concentrations, on development of fungistatic activity (B.A. units/ml.) in Penicillium gladioli cultures

Nitrogen sources in these media are as follows: N, potassium nitrate; AN, ammonium nitrate; AS, ammonium sulphate; AS+M, ammonium sulphate+malate; A, ammonium tartrate.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Glucose (% w/v)</th>
<th>Days' growth</th>
<th>Mean final dry wt. of mycelium (g./culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4 6 8 11 15 18 22</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>2.5</td>
<td>6 4 2 - - - -</td>
<td>1.3</td>
</tr>
<tr>
<td>AN</td>
<td>2.5</td>
<td>4 32 16 - - - -</td>
<td>1.4</td>
</tr>
<tr>
<td>AS</td>
<td>2.5</td>
<td>4 4 2 2 2 - -</td>
<td>1.2</td>
</tr>
<tr>
<td>AS+M</td>
<td>2.5</td>
<td>6 8 2 - - - -</td>
<td>1.1</td>
</tr>
<tr>
<td>A</td>
<td>2.5</td>
<td>6 64 32 12 - -</td>
<td>1.2</td>
</tr>
<tr>
<td>N</td>
<td>10.0</td>
<td>6 12 24 32 48 96</td>
<td>3.3</td>
</tr>
<tr>
<td>AN</td>
<td>10.0</td>
<td>4 16 16 32 32 48</td>
<td>3.9</td>
</tr>
<tr>
<td>AS</td>
<td>10.0</td>
<td>4 6 2 2 2 2 2 2</td>
<td>2.2</td>
</tr>
<tr>
<td>AS+M</td>
<td>10.0</td>
<td>4 32 32 64 96 32 24</td>
<td>3.2</td>
</tr>
<tr>
<td>A</td>
<td>10.0</td>
<td>2 8 12 16 24 32 48</td>
<td>5.8</td>
</tr>
</tbody>
</table>

which the pH does not rise too rapidly, quite high assays were recorded, though activity fell rapidly as soon as pH 6.0 was reached. It remains generally true, therefore, that to maintain high assays a high sugar concentration in the medium is necessary.

The effect of malic acid supplements in making ammonium sulphate media favourable for gladiolic acid production and accumulation was also studied. To an ammonium sulphate medium malic acid supplements were added in the range 0.05-1.0% (w/v), all media being adjusted to pH 5.5 with KOH. From the results of assays and pH drifts in P. gladioli cultures on these media (Table 4 and Fig. 5) it will be seen that the medium without supplement shows a slow fall in pH and that as the concentration of added malic acid is increased the slow fall was replaced by a rapid rise. The assays show similar relations to pH as were observed in previous experiments. The optimum medium is that with 0.25% malic acid, which produced the gradual rise in pH previously found to be favourable.

It has been shown (Brian et al. 1947) that malate supplements to an ammonium sulphate medium greatly stimulate growth of Metarrhizium glutinosum Pope (= Myrothecium verrucaria (Alb. & Schw.) Ditm. ex Fr.) and production of the antibiotic by that mould. The action of malate was attributed mainly to its effect on ammonia assimilation, and its action on the pH drift in the medium was regarded as secondary. Growth on unsupplemented ammonium sulphate
media was negligible and small concentrations of malate, or certain other 2- to 5-carbon acids, greatly stimulated growth of *M. verrucaria* under conditions where the pH drift was not significantly affected. The behaviour of *P. gladioli* is not considered to be parallel, since growth, which is quite vigorous

![Fig. 4. Effect of nitrogen source on pH drift in cultures of *Penicillium gladioli* (a) 2.5 % glucose, (b) 10.0 % glucose. (×, KNO$_3$; ○, NH$_4$NO$_3$; Δ, (NH$_4$)$_2$SO$_4$; □, ammonium tartrate; +, (NH$_4$)$_2$SO$_4$ + malate.)](image)

on unsupplemented ammonium sulphate media, is not affected; malate only affects production of gladiolic acid considerably in concentrations markedly affecting the pH drift and the effect of malate can be explained entirely by its effect on pH drift.

We conclude that the main factor influencing gladiolic acid production and accumulation in cultures of *P. gladioli* is the pH drift of the medium. Gladiolic acid accumulation is not favoured by a continued low level of pH; it is favoured
Gladiolic acid

by an upward drift of pH, but once the pH drift of the medium reaches a level above pH 6.0 the gladiolic acid is progressively destroyed. The significance of these relations is discussed below in connexion with the toxicity and stability of gladiolic acid.

![Graph](image)

**Fig. 5.** Effect of malate supplements on pH drift in cultures of *Penicillium gladioli* on an ammonium sulphate medium. (×, no malate; Δ, 0·05 % malate; ○, 0·01 %; □, 0·25 %; +, 0·5 %; △, 1·0 %.)

**Table 4.** Effect of malate supplements in an ammonium sulphate medium on development of fungistatic activity (B.A. units/ml.) in *Penicillium gladioli* cultures

<table>
<thead>
<tr>
<th>Malic acid (% w/v)</th>
<th>4</th>
<th>8</th>
<th>12</th>
<th>15</th>
<th>19</th>
<th>Mean final dry wt. of mycelium (g./culture)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>—</td>
<td>2</td>
<td>2·0</td>
</tr>
<tr>
<td>0·05</td>
<td>8</td>
<td>8</td>
<td>12</td>
<td>8</td>
<td>12</td>
<td>2·0</td>
</tr>
<tr>
<td>0·1</td>
<td>24</td>
<td>24</td>
<td>24</td>
<td>16</td>
<td>24</td>
<td>2·0</td>
</tr>
<tr>
<td>0·25</td>
<td>16</td>
<td>192</td>
<td>192</td>
<td>96</td>
<td>64</td>
<td>2·1</td>
</tr>
<tr>
<td>0·5</td>
<td>24</td>
<td>96</td>
<td>96</td>
<td>48</td>
<td>24</td>
<td>2·2</td>
</tr>
<tr>
<td>1·0</td>
<td>12</td>
<td>96</td>
<td>82</td>
<td>24</td>
<td>12</td>
<td>2·0</td>
</tr>
</tbody>
</table>

**Extraction and purification of gladiolic acid**

Samples of a filtrate from Raulin-Thom cultures, of final pH 4·4 and activity of 48 B.A. units/ml. were extracted with n-butanol (2 × 0·2 vol.), chloroform (8 × 0·1 vol.), ether (2 × 0·2 vol.) and light petroleum (b.p. 40–60°) (8 × 0·1 vol.). Of these solvents chloroform was the most effective, but not more than 50 % of the activity was removed. Treatment with activated charcoal (British Drug
Houses Ltd.) at 5 g./l. removed all activity. The active material could be recovered from the charcoal by elution with acetone, ether, methanol, ethanol or chloroform. Acetone was the most effective in that relatively small volumes were needed, but it also removed considerable quantities of pigment which made further purification more difficult. Ether was chosen as eluent; it was almost as effective as acetone and did not extract appreciable quantities of pigment. Elution was most satisfactory in a Gallenkamp Universal Extractor, in which it was consistently more rapid than in the usual Soxhlet extractor (cf. the elution of glutinosin from charcoal; Brian, et al. 1947). On evaporation of the ethereal eluate a yellow pasty mass was obtained, from which pure gladiolic acid, in the form of long, colourless, silky needles, was obtained by several recrystallizations from water.

The pH of extraction is important. A Raulin-Thom culture filtrate, assaying at 32 B.A. units/ml., was divided and samples adjusted to pH 2·0, 3·0, 4·0 and 5·4 before treatment with charcoal. Yields were respectively 0, 188, 154 and 86 mg./l. Better extraction at pH 3·0 or 4·0 than at 5·4 could be expected, but the failure of extraction at pH 2·0 cannot at present be explained. For routine production purposes all culture filtrates are now adjusted to pH 4·0 before extraction.

With Raulin-Thom medium (7·5 % glucose) yields ranged from 150 to 350 mg./l.; with Czapek-Dox (7·5 % glucose) from 150 to 200 mg./l.; with medium AN (7·5 % glucose) from 100 to 250 mg./l. Yields of the order of 300 mg./l. can now be consistently obtained on Raulin-Thom medium (7·5 % glucose) of initial pH 5·0.

**Biological activity of gladiolic acid**

**Fungistatic activity.** The least concentration of gladiolic acid required to produce 95–100 % inhibition of germination of spores of a number of fungi in Czapek-Dox medium at pH 3·5 is given in Table 5. There is a wide difference in susceptibility among the fungi tested, the lethal dose varying from 0·9 μg./ml. for *Fusarium graminearum* to 250·0 μg./ml. for *Trichoderma viride*.

<table>
<thead>
<tr>
<th>Fungus</th>
<th>Least inhibiting concentration (μg./ml.)</th>
<th>Fungus</th>
<th>Least inhibiting concentration (μg./ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Absidia glauca</em> Hagem</td>
<td>1·9</td>
<td><em>Penicillium digitatum</em> Sacc.</td>
<td>15·6</td>
</tr>
<tr>
<td><em>Aspergillus flavus</em> Link</td>
<td>125·0</td>
<td><em>P. expansum</em> Link</td>
<td>3·9</td>
</tr>
<tr>
<td><em>A. niger</em> van Tiegh.</td>
<td>125·0</td>
<td><em>P. gladioli</em> McCull. &amp; Thom</td>
<td>7·8</td>
</tr>
<tr>
<td><em>Botrytis allii</em> Munn.</td>
<td>7·8</td>
<td><em>P. jancezewskii</em> Zal.</td>
<td>1·9</td>
</tr>
<tr>
<td><em>Byssoschlamys fulva</em> Olliver &amp; Smith</td>
<td>1·9</td>
<td><em>P. notatum</em> Westling</td>
<td>3·9</td>
</tr>
<tr>
<td><em>Cephalosporium longisporum</em> Petch</td>
<td>15·6</td>
<td><em>Stemphylium</em> sp.</td>
<td>62·5</td>
</tr>
<tr>
<td><em>Fusarium caeruleum</em> (Lib.) Sacc.</td>
<td>3·9</td>
<td><em>Thamnidium elegans</em> Link</td>
<td>62·5</td>
</tr>
<tr>
<td><em>F. graminearum</em> Schwabe</td>
<td>0·9</td>
<td><em>Trichoderma viride</em> Pers. ex Fries</td>
<td>250·0</td>
</tr>
<tr>
<td><em>Myrothecium verrucaria</em> (Alb. &amp; Schw.) Ditm. ex Fries</td>
<td>62·5</td>
<td><em>Trichothecium roseum</em> Link</td>
<td>250·0</td>
</tr>
<tr>
<td><em>Mucor erectus</em> Bain.</td>
<td>7·8</td>
<td><em>Verticillium alboatrum</em> Reinke &amp; Berth.</td>
<td>7·8</td>
</tr>
</tbody>
</table>
Gladiolic acid

The fungistatic activity of gladiolic acid varies with the pH of the solution, as is usually the case with weak acids. In Table 7, the fungistatic activity of a 0.2% solution of gladiolic acid at pH 3.0 is 512 B.A. units/ml., falling to only 2 B.A. units/ml. at pH 7.0. These results are best explained on the assumption that it is only the lipid-soluble, undissociated gladiolic acid molecules which penetrate the plasma membrane of the spore.

It will be noted that gladiolic acid is quite toxic to P. gladioli at pH 3.5. This suggests an explanation of the increased gladiolic acid production in cultures with a rising pH; if the pH remained level near pH 4.0, a concentration of gladiolic acid toxic to P. gladioli (c. 10 mg./l.) would very soon be reached, but with a rising pH increased dissociation of gladiolic acid molecules allows the accumulation of much higher concentrations (greater than 300 mg./l.) without any undue autotoxic effect.

**Antibacterial activity.** When included in broth at pH 7.0 gladiolic acid exerts little bacteriostatic effect. Growth of Staphylococcus aureus (two strains), Salmonella typhi, Bacterium lactis aerogenes, B. subtilis, B. brevis, Escherichia coli and Micrococcus lysodeikticus were not inhibited by 500 µg./ml. gladiolic acid. The other strains of S. aureus were inhibited at 250 µg./ml.

Nevertheless, under certain conditions, gladiolic acid is markedly bactericidal. S. aureus (N.R.R.L. 313), Salmonella typhi (N.C.T.C. 786) and Esch. coli (N.C.T.C. 419) were suspended, at 20°, in broth adjusted to pH 4.0 and 7.0 and in citric acid-phosphate buffers at pH 4.0 and 7.0, containing in each case gladiolic acid at 10 and 100 µg./ml. Loopfuls were taken out after 6, 12, 18, 24, 30, 45 and 60 min. and streaked on nutrient agar. There was no bactericidal effect with either concentration of gladiolic acid in broth at either pH. The times taken for 100 µg./ml. gladiolic acid to produce a complete, or virtually complete, kill in buffer at pH 4.0 were Staph. aureus 18 min., Salmonella typhi 12 min., Esch. coli 18 min. In buffer at pH 7.0 the corresponding times were: Staph. aureus > 60 min., S. typhi 18 min., Esch. coli 45 min. Gladiolic acid at 10 µg./ml. in buffer at pH 4.0 caused some kill of S. typhi in 60 min. but did not affect the other organisms; at pH 7.0 this concentration had no effect on any of the organisms. Thus the antibacterial effect of gladiolic acid is related to pH, as was the antifungal effect, and its antibacterial activity is reduced by some constituent of broth (see below).

**Stability of gladiolic acid solutions in relation to pH**

Gladiolic acid solutions (0.2%) in Weindling and Czapek-Dox media at pH 3.0, 5.0, 7.0 and 8.0 were assayed after various heat treatments. All solutions were readjusted to pH 3.5 before assay. The results presented in Table 6 indicate (a) that in either medium at pH 3.0 gladiolic acid is relatively stable, (b) that in Weindling medium at pH 5.0 or above gladiolic acid is rapidly inactivated even in the cold, but (c) that in Czapek-Dox it is much more stable and even at pH 8.0 severe heat treatment is necessary to produce any considerable loss in activity.

In the Weindling medium loss in activity is associated with the formation of
a green precipitate; it was recorded previously (Brian et al. 1946) that gladiolic acid gives a green colour when treated with ammonia, and it therefore seemed probable that the inactivation of gladiolic acid in Weindling was due to reaction with ammonium tartrate. Czapek-Dox contains no ammonium salts, nitrogen being supplied as nitrate.

Table 6. Effect of heat treatments on fungistatic activity of gladiolic acid solutions

<table>
<thead>
<tr>
<th>pH of solution</th>
<th>No treatment (min.)</th>
<th>Autoclaved at 120° (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>(a) In Czapek-Dox medium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>255</td>
<td>192</td>
</tr>
<tr>
<td>5.0</td>
<td>256</td>
<td>192</td>
</tr>
<tr>
<td>7.0</td>
<td>256</td>
<td>192</td>
</tr>
<tr>
<td>8.0</td>
<td>256</td>
<td>64</td>
</tr>
</tbody>
</table>

(b) In Weindling medium

<table>
<thead>
<tr>
<th>pH of solution</th>
<th>8.0</th>
<th>7.0</th>
<th>5.0</th>
<th>3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>256</td>
<td>128</td>
<td>128</td>
<td>192</td>
</tr>
<tr>
<td>7.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>5.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 7. Activity of 0.2% gladiolic acid solutions in Czapek-Dox (a) assayed after readjustment to pH 8.5, (b) assayed at pH of solution

<table>
<thead>
<tr>
<th>pH of solution</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Assayed at pH 8.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>256</td>
<td>192</td>
<td>192</td>
<td>256</td>
<td>192</td>
</tr>
<tr>
<td>4.0</td>
<td>256</td>
<td>192</td>
<td>128</td>
<td>192</td>
<td>256</td>
</tr>
<tr>
<td>5.0</td>
<td>256</td>
<td>128</td>
<td>192</td>
<td>128</td>
<td>192</td>
</tr>
<tr>
<td>6.0</td>
<td>256</td>
<td>128</td>
<td>192</td>
<td>128</td>
<td>192</td>
</tr>
<tr>
<td>7.0</td>
<td>256</td>
<td>192</td>
<td>128</td>
<td>192</td>
<td>256</td>
</tr>
<tr>
<td>8.0</td>
<td>256</td>
<td>192</td>
<td>128</td>
<td>192</td>
<td>192</td>
</tr>
</tbody>
</table>

(b) Assayed at pH of solution

<table>
<thead>
<tr>
<th>pH of solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
</tr>
<tr>
<td>4.0</td>
</tr>
<tr>
<td>5.0</td>
</tr>
<tr>
<td>6.0</td>
</tr>
<tr>
<td>7.0</td>
</tr>
<tr>
<td>8.0</td>
</tr>
</tbody>
</table>

At physiological temperatures gladiolic acid is very stable in Czapek-Dox between pH 3.0 and 8.0 (Table 7). Similar experiments indicated that at pH 8.5 or below it is quite stable in Weindling medium. Table 7 also shows that the activity of gladiolic acid is dependent on the pH of the solution.
Inactivation of gladiolic acid by ammonium salts and amino-acids

Ammonium salts. The suspected role of ammonium salts in the inactivation of gladiolic acid in Weindling medium was confirmed and the inactivation studied by periodical assays of mixtures of gladiolic acid and ammonium chloride in McIlvaine's citric acid-phosphate buffer (Table 8). It will be seen that (a) the inactivation is dependent on pH, proceeding at pH 5·0 and even more rapidly at pH 7·0 but not at pH 3·3, (b) the process is relatively slow at the temperature chosen (25°), and (c) even at the most favourable pH level an excess of ammonium chloride molecules is needed to produce complete inactivation, though activity drops to 25% of the original where ammonium chloride and gladiolic acid are present in equimolecular proportions. In all cases where appreciable inactivation took place yellow or green colours developed in the solutions.

Amino-acids. In their studies on glyoxalase, Dakin & Dudley (1918, 1914) recorded a reaction between phenyl glyoxal and ammonia, giving coloured products and between phenyl glyoxal and certain amino-acids, notably histidine, arginine, ornithine and lysine, to give sparingly soluble yellow substances. Accordingly, in view of the structural relationship between gladiolic acid and phenyl glyoxal, the activity of gladiolic acid in the presence of certain amino-acids has been studied (Table 9). Several amino-acids inactivated gladiolic acid, the most effective, in descending order, being p-aminobenzoic acid, histidine, tryptophan, arginine and glycine. In all cases where marked inactivation took place yellow or green colours were produced. p-Aminobenzoic acid was outstanding, producing immediate and complete inactivation when mixed in equimolecular proportions with gladiolic acid; it did not completely
inactivate when present in less than equimolecular proportions. \( p \)-Aminobenzenesulphonamide was equally effective; anthranilic (\( o \)-aminobenzoic acid) was somewhat less effective and the results were complicated by the marked fungistatic activity of this compound.

**Table 9. Fungistatic activity of mixtures of gladiolic and various amino-acids in Czapek-Dox (pH 3.5), assayed immediately and after 24 hr. storage at 25°**

The 0.001 M gladiolic acid alone showed an activity of 16 B.A. units/ml; none of the amino-acids alone showed fungistatic activity at 0.01 M, neither did they stimulate.

<table>
<thead>
<tr>
<th>Amino-acid</th>
<th>0.001 M gladiolic acid + 0.01 M amino-acid</th>
<th>0.001 M gladiolic acid + 0.001 M amino-acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr.</td>
<td>24 hr.</td>
</tr>
<tr>
<td></td>
<td>B.A. units/ml</td>
<td>B.A. units/ml</td>
</tr>
<tr>
<td>DL-Alanine</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>( p )-Aminobenzoic acid</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>L-Arginine hydrochloride</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>12</td>
<td>4</td>
</tr>
<tr>
<td>Glycine</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>L-Histidine hydrochloride</td>
<td>---</td>
<td>12</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>L-Proline</td>
<td>16</td>
<td>8</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>8</td>
<td>12</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>12</td>
<td>8</td>
</tr>
</tbody>
</table>

**Discussion**

The inactivation of gladiolic acid by ammonium salts and amino-acids may be of significance in explaining the disappearance of gladiolic acid from cultures of *P. gladioli* when the pH rises above 6.0 and the mechanism of the antifungal action of gladiolic acid.

Cultures of *P. gladioli* produce gladiolic acid most abundantly in circumstances where, after an initial fall, there is a rising trend in pH, but that after pH 6.0 or thereabouts is reached gladiolic acid rapidly disappears. As has been shown, gladiolic acid is intrinsically stable under such conditions, but also does react with ammonium salts at that pH, forming an insoluble inactive compound. Ammonium salts are normally present in cultures after several days’ incubation, even if nitrogen is originally supplied as nitrate, so there is every opportunity for this kind of reaction to occur; in fact dark green precipitates, similar in appearance to those produced by reaction of ammonium salts with gladiolic acid, were observed in cultures, particularly frequently when nitrogen was supplied as ammonium nitrate.

The inactivation of gladiolic acid by certain amino-acids, notably \( p \)-aminobenzoic acid, histidine and tryptophan, suggests that its mode of action may be concerned with immobilization of such amino-acids in the fungal cell. This
Gladiolic acid possibility will be investigated. It is not suggested that the inactivation of gladiolic acids by amino-acids is in any strict sense specific; it is highly probable that many aromatic amines would have a similar effect.

We wish to thank Miss Valerie Spence, Mr G. W. Elson and Mr C. H. Unwin for much valuable assistance in this investigation.

REFERENCES


(Received 19 March 1948)